Subunit Structure and Amino Acid Composition of Mouse Submaxillary Gland Nerve Growth Factor*

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ABSTRACT: The subunit structure and amino acid composition of mouse submaxillary gland nerve growth factor (NGF) have been examined. Sedimentation equilibrium analyses of the protein in 0.1 N sodium acetate (pH 5.0) yielded a molecular weight of 29,000. In unbuffered 6 M guanidine hydrochloride (Gd-HCl), NGF undergoes apparent dissociation to half-molecules of mol wt 14,500. Reduction and alkylation of the disulfide bonds do not further reduce the molecular weight in Gd-HCl. Amino-terminal analyses by the cyanate method indicated that the native protein has two residues of serine as amino end groups. Fractionation of the soluble tryptic peptides of *S*-[^{14}C]carboxymethyl NGF, by column chromatography and one-dimensional paper electrophoresis, resulted in the isolation of 15 peptides containing 5 residues of *S*-carboxymethylcysteine. Digestion and fractionation of the acid-insoluble portion of the tryptic digest with thermolysin, yielded one additional peptide containing a single residue of *S*-carboxymethylcysteine. On the basis of the lysine and arginine content of native NGF, 35 tryptic peptides, containing 12 residues of *S*-carboxymethylcysteine could be maximally expected. These results suggest that mouse NGF is composed of two very similar or identical subunits of 14,500 molecular weight that are associated in the native molecule by noncovalent forces.

Nerve growth factor (NGF)[1] is a compound which elicits the overgrowth of the sympathetic chain ganglia *in vivo* and a halo-like outgrowth of nerve fibers from embryonic sympathetic or sensory ganglia cultured *in vitro*. The biological significance of this factor has been emphasized by the demonstration that inhibition of its action by antibodies specific for purified NGF results in the total destruction of the sympathetic nervous system (Levi-Montalcini and Angeletti, 1966, 1968).

Although this factor is widely distributed in a variety of tissues in trace amounts, molecular studies have been limited to the NGF isolated from snake venoms and male mouse submaxillary glands, where it is found in relatively large quantities. Isolation of NGF from the latter source, first reported by Cohen (1960), indicated that the biological activity was associated with a protein moiety of mol wt 44,000. By a modified procedure, Bocchini and Angeletti (1969) have obtained preparations with a molecular weight of only 30,000 and Zanini *et al.* (1968) have reported the isolation of a fully active protein of only 14,000 molecular weight. Significantly, the immunochemical properties of this latter species were indistinguishable from those of the 30,000 molecular weight form. These observations, which suggest a polymeric structure for mouse NGF as isolated by Cohen and by Bocchini and Angeletti, are inconsistent with an amino acid composition of 5 residues of cystine per 30,000 molecular weight, as reported by Bocchini (1970).

In view of this discrepancy, the subunit structure and amino acid composition of mouse submaxillary NGF have been reexamined. The results reported here, which form the basis for a detailed examination of the structure–function relationships of this protein, indicate that mouse NGF, as isolated, is composed of two very similar, or identical, subunits of approximately 14,500 molecular weight.

Experimental Section

Materials. NGF was purified from male mouse submaxillary glands as described by Bocchini and Angeletti (1969). Samples of NGF, usually prepared in about 15-mg quantities, were routinely checked by disc gel electrophoresis at pH 4.3 before use.

Trypsin, treated with TPCK, was purchased from Worthington Biochemical Corp. Thermolysin was obtained from Daiwa Kasei K. K., Osaka, Japan.

Gd-HCl, used in the sedimentation equilibrium experiments, was the Spectrophotometric grade from Heico, Inc. In all other experiments, Ultra Pure Gd-HCl from Mann Research Laboratories was used. Potassium cyanate for amino-terminal analyses was purchased from Fisher Chemical Co. Dithiothreitol was obtained from Nutritional Biochemicals. The *p*-mercuribenzoic acid, used in the sulfhydryl group titrations, was purchased from Mann Research Laboratories.

Pyridine and *N*-ethylmorpholine were redistilled after the addition of solid ninhydrin (1 g/l.) to the distillation flask.

Crystalline [1-^{14}C]iodoacetic acid with a specific activity of 14.9 mCi/mmole was obtained from New England Nuclear Corp. Unlabeled iodoacetic acid was recrystallized from cold petroleum ether (bp 30–60°) before use.

Methods. Radioactive *S*-carboxymethyl NGF was prepared in the following manner. NGF (50 mg, 1.7 μmoles),

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[1] Abbreviations used are: NGF, nerve growth factor; M_w, weight-average molecular weight; Gd-HCl, guanidine-HCl; $\bar{v}$, partial specific volume; TPCK, (1-tosylamido-2-phenyl)ethyl chloromethyl ketone; Tp-, tryptic peptide; Th-, thermolytic peptide.

dissolved in 2 ml of 6 M Gd-HCl (pH 9.3), was reduced using a 50-fold molar excess of dithiothreitol to protein in a nitrogen atmosphere for 4 hr. After adjusting the protein solution to pH 8.0 with 1 N HCl, a neutral solution containing 2020 μmoles of unlabeled iodoacetic acid and 3.2 μmoles of radioactive iodoacetic acid was added. Alkylation was allowed to proceed for 5 min. The reaction was stopped by addition of a 10-fold molar excess of 2-mercaptoethanol. After dialysis against four changes of deionized water for 24 hr, the radioactive *S*-carboxymethyl NGF was recovered by lyophilization. Unlabeled *S*-carboxymethyl NGF was prepared in a similar manner.

Radioactivity was measured in a Packard Model 3370 liquid scintillation spectrometer with a counting efficiency for ^{14}C of 84%. Aliquots for analyses were dissolved in 10 ml of Bray's water miscible scintillation liquid (1960). Radioactivity in the column chromatography experiments was measured with 0.1-ml aliquots withdrawn from alternate fractions.

Tryptic digestion was carried out using TPCK-treated trypsin added in a ratio of 1:100 to the NGF on a weight basis according to Bradshaw *et al.* (1969a). [^{14}C]*S*-Carboxymethyl NGF (30 mg, 1.03 μmoles) was used in the preparation of the tryptic fingerprint. The reaction mixture was maintained at pH 8.8, 37° by means of a Radiometer pH-Stat, Model TTT-11. The insoluble material remaining after acidification to pH 2 with 6 N HCl was removed by centrifugation.

The soluble tryptic (Tp) peptides were fractionated on a 0.9 × 20 cm column of Dowex 50-X8 equilibrated in 0.05 N pyridine acetate (pH 2.5), and eluted with a double linear gradient of pyridine acetate buffers as described by Bradshaw *et al.* (1969b). The separation of peptides was monitored with a Technicon AutoAnalyzer equipped for automatic alkaline hydrolysis and ninhydrin analysis (Hill and Delaney, 1967). The isolated peptides were recovered after removal of the solvent by rotary evaporation. High-voltage paper electrophoresis on all Tp pools was performed on Whatman No. 1 paper in pyridine acetate buffer (pH 3.75) at 2000 V for 1.5 hr. Peptides were detected by ninhydrin reagent and tryptophan-containing peptides were identified by Ehrlich's reagent (Smith, 1953).

The insoluble material remaining after tryptic digestion was suspended in 3 ml of water and brought to pH 8.0 with 2 N NaOH. Digestion with thermolysin was carried out as described by Bradshaw (1969) at pH 8.0, 37°. The insoluble material remaining after adjusting the digestion mixture to pH 9.0 with 1 N NaOH was removed by centrifugation. The soluble thermolytic peptides (denoted as Tp/Th) were separated on a column of Dowex 1-X2, 0.9 × 150 cm, equilibrated in 3% pyridine as described previously (Bradshaw *et al.*, 1969b).

Amino acid analyses were performed on a Beckman 120C automatic amino acid analyzer by the method of Moore *et al.* (1958). The tryptophan content of the native protein was determined spectrophotometrically by the method of Edelhoch (1967). Half-cystine was determined as cysteic acid (Moore, 1963) and as *S*-carboxymethylcysteine on the automatic amino acid analyzer. The number of free sulfhydryl groups was estimated by titration with *p*-mercuribenzoate in 8 M urea as described by Boyer (1954). Amino end-group analysis was performed by the quantitative hydantoin method of Stark and Smyth (1963). As a control, a sample of NGF not treated with potassium cyanate or urea was prepared in parallel to the carbamyl NGF. The concentration of protein in the experiment and in the control was estimated from an acid hydrolysate of an aliquot removed from the samples prior to cyclization.

Sedimentation equilibrium experiments were performed using a Spinco Model E ultracentrifuge, following the high-speed technique of Yphantis (1964). Samples were dissolved in the appropriate solvent (either 0.1 N sodium acetate, pH 5.0, or unbuffered 6 M Gd-HCl, pH 3.3, density 1.1444) and dialyzed overnight before each analysis. Solutions of differing concentration, as judged by absorbance at 280 mμ, for each of the three sectors of the cell were made by dilution of the dialyzed stock solution with dialysate. The concentration of protein in milligrams per milliliter was calculated from the relation: $\epsilon_{280\ nm}^{1\%,\ 1\ cm} = 13.8$ (Bocchini, 1970). Analyses were performed at 20° and 48,000 rpm. Photographic plates, made after 18–22 hr at speed, were read manually using a microcomparator and the data processed on a CDC 6400 digital computer, using the programs of Teller *et al.* (1969).

Weight-average molecular weights, M_w, were calculated from the relation

$$M_w = \frac{C_b - C_m}{\frac{C_b}{M_{n,b}} - \frac{C_m}{M_{n,m}}}$$

where

$$M_n = \frac{\int_m^b c\,d(r^2)}{\int_m^b \frac{c}{M_{n,r}}\,d(r^2)}$$

and C_m is the concentration of protein at the meniscus, C_b is the concentration of protein at the bottom of the cell, $M_{n,m}$ is the number-average molecular weight at the meniscus, $M_{n,b}$ is the number-average weight at the bottom of the cell, $M_{n,r}$ is the number-average molecular weight as a function of the radius, c is the protein concentration, and r is the radius from the center of rotation.

When C_m is negligible, the term $C_m/M_{n,m}$ is less than 1% of the $C_b/M_{n,b}$ term and $M_w \cong M_{n,b}$ and $M_z \cong M_{w,b}$.

These are whole cell average values. Weight-average molecular weights are computed as the average values, on a point-by-point basis, of two plots, *i.e.*, ln c *vs.* r^2 and $\int c\,d(r^2)$ *vs.* c.

The partial specific volume, $\bar{v}$, was calculated from the amino acid composition.

Results

Molecular Weight Determinations. Previous sedimentation equilibrium analyses by Bocchini and Angeletti (1969) indicated that mouse NGF was a pure protein of mol wt 30,000. Gel filtration experiments on the same material (Zanini *et al.*, 1968) gave values of 28,000, in close agreement with that obtained from the ultracentrifuge. In one experiment, however, Zanini *et al.* observed that, upon standing in dilute solution for a period of 7 days, NGF underwent an apparent partial dissociation resulting in the elution of the biological activity from the column in two peaks. By calibration of the column these samples were estimated to have mol wt 28,000 and 14,000.

In view of these results, a reexamination of the molecular weight of mouse NGF by sedimentation equilibrium in the

TABLE I: Molecular Weight of Mouse Nerve Growth Factor by Sedimentation Equilibrium Analysis.[a]

Sample	Solvent	Expt	Protein Concn (mg/ml)[b]	M_w
Native NGF	0.1 M sodium acetate (pH 5.0)	1	0.62	29,796
		2	0.72	29,230
		3	0.90	27,894
		Average		28,973 ± 976
Native NGF	6 M Gd-HCl (pH 3.3)	1	0.37	15,643
		2	0.52	16,813
		Average		16,228 ± 585
S-Carboxymethyl NGF	6 M Gd-HCl (pH 3.3)	1	0.28	16,901
		2	0.37	15,364
		3	0.70	13,273
		4	0.37	14,532
		5	0.52	14,037
		6	0.73	13,074
		Average		14,530 ± 1432

[a] $\bar{v}$ = 0.716 ml/g. [b] Determined spectrophotometrically, $\epsilon_{280nm}^{1\%,1cm}$ 13.8 (Bocchini, 1970).

ultracentrifuge in buffer and in 6 M Gd-HCl was undertaken. The results of these experiments are summarized in Table I. For native NGF in 0.1 M sodium acetate (pH 5.0), a weight-average molecular weight (M_w) of 28,973 ± 976, averaged from the three concentrations, was obtained. This result is in excellent agreement with that of Bocchini and Angeletti (1969) and Zanini *et al.* (1968). The molecular weight of 16,225 ± 585, obtained in 6 M Gd-HCl, however, is considerably lower and approaches a value half that of the native molecular weight. In order to ascertain whether the molecular weight of NGF could be further reduced by the scission of the six disulfide bonds present in the native molecule (*vide infra*), native NGF was reduced and alkylated in 6 M Gd-HCl with iodoacetic acid. The resultant *S*-carboxymethyl NGF was subjected to similar ultracentrifugal analyses in 6 M Gd-HCl. In this case, as indicated in Table I, six determinations were made, yielding an average M_w of 14,530 ± 1432. This value is exactly half of the native molecular weight and within experimental error of the value determined for native NGF in 6 M Gd-HCl. These data suggest a dimeric structure for the protein composed of two polypeptide chains of equal, or nearly equal, molecular weight.

End-Group Analysis. The amino-terminal residue(s) of mouse NGF were determined by the cyanate method of Stark and Smyth (1963). The results of these experiments are shown in Table II. The values for the control samples were subtracted from the end-group values to give the net values which were finally corrected by the destruction factors experimentally determined by Stark and Smyth (1963). A molecular weight of 29,000 for NGF was used in these calculations.

Four amino acids, arginine, threonine, serine, and glutamic acid, were found to be present in appreciable quantities. Only serine, however, was present in amounts consistent with an integral end group. The amount of glutamic acid, prior to correction for destruction, is comparable to that observed in other end-group determinations, performed by this method, and most likely arises from incomplete removal of pyrrolidonecarboxylic acid formed during the cyclization reaction. In a like manner, even after correction for destruction, the amounts of threonine and arginine represent considerably less than stoichiometric quantities. It should be noted, particularly in the case of threonine and glutamic acid, that the destruction factor is only valid for the conversion of the hydantoin into the corresponding amino acid, and will produce erroneously high values for contaminating free amino acids.

An unknown peak, which chromatographed on the automatic amino acid analyzer prior to aspartic acid and which did not elute in the position of any reported amino acid or amino acid derivative was also observed. In view of its apparent low yield, it was not considered to be a major end group.

The results of these experiments which indicate the presence of two residues of serine per molecule of native NGF as amino terminal support the previous conclusions regarding

TABLE II: Amino-Terminal Analysis of Mouse Nerve Growth Factor.

Amino Acid	Residues Recov/ 29,000 g	Control	Cor End Group[a]
Lysine	0.06		0.07
Histidine	0.20	0.18	0.03
Arginine	0.63	0.37	0.34
Unidentified[b]	(0.26)		(0.26)
Threonine	0.07		0.25
Serine	0.51	0.04	2.35
Glutamic acid	0.29		0.48
Glycine	0.10	0.06	0.05
Alanine	0.04		0.04
Valine	0.02		0.03
Methionine	0.16		0.17

[a] Corrected by the factors determined by Stark and Smyth (1963). [b] Calculated using the color constant of aspartic acid.

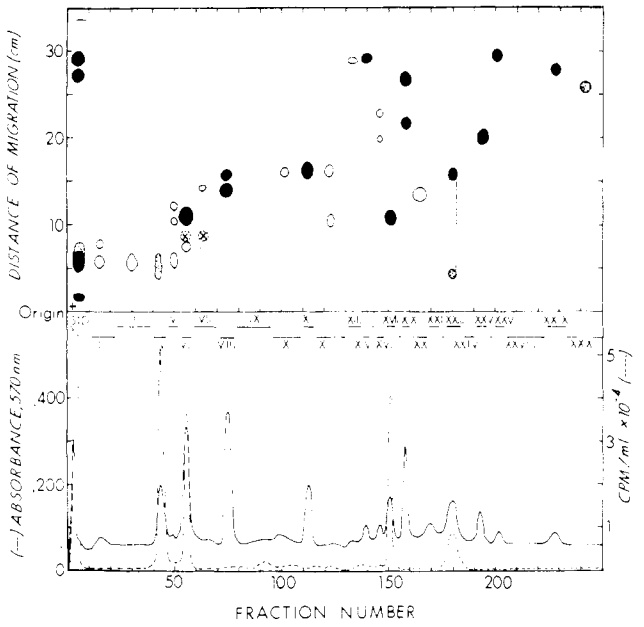

FIGURE 1: Elution profile and high-voltage electrophoresis of soluble tryptic peptides of 30 mg (1.03 μmoles) of [^{14}C]*S*-carboxymethyl NGF on a 0.9 × 20 cm column of Dowex 50-X8 at 55°. The column was developed at 30 ml/hr with a double linear gradient of pyridine acetate, as described in the text and monitored automatically by ninhydrin analysis (——) after alkaline hydrolysis. Fractions of 3.0 ml were collected. Radioactivity (---) was counted on 100-μl aliquots taken from alternate fractions. Fractions were pooled as indicated by the bars and corresponding Roman numerals. The electrophoresis was carried out at pH 3.75, 2000 V for 1 hr. The ninhydrin-positive patterns for each pool are shown above the corresponding bar. The shading of the peptides indicates the relative intensity: solid, heavy; dotted, medium; open, light; dashed with x, ninhydrin negative and tryptophan positive; STD, standard amino acid mixture.

TABLE III: Recovery of Radioactive Tryptic Peptides of [^{14}C]*S*-Carboxymethyl NGF.

	Total cpm	μmoles[a] of Peptide (A)	μmoles[b] of *S*-CM-Cys (B)	Ratio B/A
Soluble tryptic fraction	4,300,000			
Tp-IV	430,578	1.53	2.60	1.7
Tp-VI	238,492	1.76	1.58	0.9
Tp-XVII	208,830	1.47	1.38	0.94
Tp-XXIII	123,504	0.88	0.84	0.95
S-Carboxymethyl-2-mercaptoethanol	1,700,000			
Total soluble cpm recovered	2,700,000			
% recovery (soluble fraction)	63			

[a] Micromoles of peptide were calculated from the quantity of lysine or arginine of each purified pool after amino acid analysis. [b] Micromoles of *S*-carboxymethylcysteine in soluble tryptic peptides were calculated from amino acid analysis of each pool.

the dimeric nature of this protein, and further suggest that the subunits may have similar or identical chemical structures.

Column Fingerprint of the Soluble Tryptic Peptides of [^{14}C]-*S-Carboxymethyl NGF.* In view of the results obtained from the sedimentation equilibrium and the amino-terminal analyses, an examination of the tryptic peptides was undertaken to further ascertain the number and kind of polypeptide chains present in native NGF. On the basis of the amino acid composition (*vide infra*) which assigns 19 residues of lysine and 15 residues of arginine per 29,000 molecular weight, 35 tryptic peptides may be expected. If the molecule is composed of two identical subunits, only half this number plus one[2] should be obtained rather than the 35 unique peptides expected from a monomeric structure or one composed of two dissimilar polypeptide chains. In order to permit an exact accounting of the number and the distribution of the half-cystinyl residues, the protein was first reduced and alkylated with ^{14}C-labeled iodoacetate. By the same reasoning, only six unique radioactive peptides should be recovered from a dimer of identical chains.

The separation of the tryptic hydrolysate was carried out on a column of Dowex 50-X8 with a double linear gradient of pyridine acetate followed by one-dimensional electrophoresis at pH 3.75. The elution profile for the fractionation of the soluble tryptic peptides on the ion-exchange column is shown in the lower part of Figure 1. The corresponding electrophoretogram for each of the isolated pools, indicated by the solid bars, is shown in the upper portion of the figure.

The left-hand column (standard) shows the amino acid standard, placed in the position of fraction I in this Figure, since that fraction contained no ninhydrin-positive material.[3] Fraction XXX, the last pool eluted, also contained the strip fraction, not shown in the elution profile. Examination of the elution profile shows that, with the exception of fraction I, there are 13 major peaks, of which 4 contain radioactivity. In addition, the electrophoretic analyses indicated that pools VII and XIX each contained two major peptides, and that the peaks corresponding to pools XVI and XXI contained little or no peptidic material. Pool XXX, which also contained the column strip, also had one peptide. Staining of the electrophoretograms for tryptophan yielded one additional peptide in pools VI and VII, indicated by the crosses, which did not react with ninhydrin. Thus, by combination of the data obtained from the column profile and the paper electrophoresis, 15 major peptides can be accounted for.

An analysis of the radioactivity recovered in this fractionation is summarized in Table III. Only 5 fractions, I, IV, VI, XVII, and XXIII contained significant amounts of radioactivity. Fraction I, which contained 63% of the total radioactivity recovered, was virtually devoid of peptidic material. Ninhydrin absorbance observed was caused by the cysteic acid added to the hydrolysate prior to fractionation as a column marker. The radioactivity found in this fraction, therefore, has been assigned to *S*-carboxymethyl-2-mercaptoethanol, formed at the termination of the alkylation reaction

[2] The number of potential tryptic cleavages was calculated from the lysine and arginine content of native NGF (mol wt 29,000). Because these values do not produce even integers when divided by 2, the number of tryptic peptides expected from the subunit is actually 2 less, as indicated by the lysine and arginine content of the minimal subunit (see Table V).

[3] Under the conditions used, the cysteic acid column marker, present in fraction I, migrates into the electrode chamber.

TABLE IV: Recovery of Radioactive Tryptic/Thermolytic Peptides of [^{14}C]*S*-Carboxymethyl NGF.

	Total cpm	μmole of *S*-CM-Cys[a]
Insoluble tryptic fraction	880,434	
Soluble thermolytic fraction	815,430	
Tp/Th-VIII	28,146	0.20
Tp/Th-XI	14,076	0.10
Tp/Th-XII	19,284	0.14
Tp/Th-XV	13,896	0.10
Tp/Th-XVI	19,266	0.14
Tp/Th-XX	13,296	0.10
Tp/Th-XXI	29,580	0.21
Tp/Th-XXII	10,416	0.07
Tp/Th-XXIII	6,936	0.05
Tp/Th-XXIV	11,796	0.08
Tp/Th-XXVI	28,890	0.21
Tp/Th-XXVII	44,988	0.32
Tp/Th-XXVIII	132,648	0.95
Tp/Th-XXX	25,698	0.18
All other pools, less than 0.05 μmole each	25,092	
Total cpm recovered	472,260	
% recovery (insoluble fraction)	58	

[a] Micromoles of *S*-carboxymethylcysteine in the thermolysin pools were determined from the specific activity of *S*-carboxymethylcysteine of the soluble tryptic peptides, 139,700 cpm/μmole.[4]

and which was incompletely removed by dialysis. The remaining four labeled fractions were each found to contain predominantly one peptide. Only fraction VI required purification on Dowex 1-X2 (Bradshaw *et al.*, 1969b). From amino acid analysis of these fractions, the number of micromoles of peptide, as judged by the lysine or arginine content and the number of μmoles of *S*-carboxymethylcysteine were calculated (columns 2 and 3 of Table III). The ratio of these values, shown in the fourth column, indicates that the peptide of fraction IV contains two residues of *S*-carboxymethylcysteine, whereas the other three fractions contain one each. Thus five residues of *S*-carboxymethylcysteine are accounted for in the soluble tryptic peptides. From these data, the specific activity of the radioactive label was calculated to be 139,700 cpm/μmole of *S*-carboxymethylcysteine.[4]

Column Fingerprint of the Soluble Thermolytic Peptides of the Tryptic Insoluble Fraction of [^{14}C]*S-Carboxymethyl NGF.* Although it was not readily feasible to obtain an accurate count of the number of tryptic peptides in the insoluble portion of the digest, it was possible to examine the distribution of radioactivity in this fraction by digestion with thermolysin. Despite this treatment, however, a small amount of

[4] A specific activity of 139,700 cpm/μmole of *S*-CM-cysteine was calculated by dividing the number of cpm for each pool by the number of micromoles of peptide, or in the case of fraction Tp-IV, by twice this number. The values for the four fractions were averaged. By assuming an integral amount of *S*-CM-cysteine to be present, it was not necessary to correct for the destruction of this residue during acid hydrolysis, which was found to be somewhat variable.

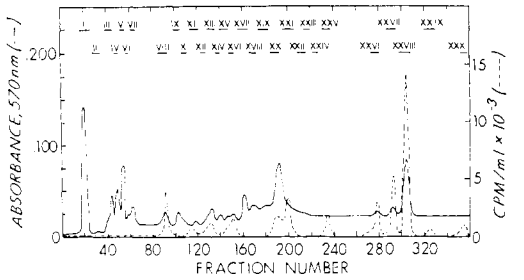

FIGURE 2: Elution profile of the soluble thermolytic peptides of the insoluble tryptic core of [^{14}C]*S*-carboxymethyl NGF on a 0.9 × 150 cm column of Dowex 1-X2 at 35°. The column was developed at 30 ml/hr with a gradient of pyridine acetate, as described in the text and monitored by automatic ninhydrin analysis (——) after alkaline hydrolysis. Fractions of 3.0 ml were collected. Radioactivity (---) was counted on 100-μl aliquots removed from alternate fractions. Fractions were pooled as indicated by the bars and corresponding Roman numerals.

insoluble material was still present at the end of the reaction. In view of the fact that 93% of the radioactivity had been solubilized, this insoluble material was not analyzed further. The soluble tryptic/thermolytic peptides were fractionated on a column of Dowex 1-X2 as shown in Figure 2. As expected, several peptides were indicated by the ninhydrin analysis, including one major and several minor radioactive peaks. The recovery of the labeled peptides from this sample is summarized in Table IV. By means of the specific activity calculated from the soluble tryptic peptides, the number of micromoles of *S*-carboxymethylcysteine present in each fraction, has been calculated. As observed above, only one radioactive fraction, Tp/Th XXVIII, contained a high yield of peptide, *i.e.*, 0.95 μmole. These results suggest that only one major half-cystinyl peptide is located in the tryptic core. The amino acid composition of this peptide excluded the possibility that it was derived from any of the regions of the molecule represented by the labeled soluble tryptic peptides.[5] Thus, five half-cystinyl peptides were found in the soluble fraction and one in the insoluble fraction. These results establish that mouse NGF contains six unique half-cystinyl residues.

Amino Acid Composition of Mouse NGF. The amino acid composition of mouse NGF was calculated from acid hydrolysates incubated for 24, 48, and 72 at 110°. In each case, a minimum of two analyses were averaged to give the final values. The values for half-cystine were calculated as cysteic acid from acid hydrolysates of protein that had been oxidized with performic acid (Moore, 1963). These results were compared to the content of *S*-carboxymethylcysteine from protein which had been reduced and alkylated. Tryptophan was determined spectrophotometrically by the method of Edelhoch (1967). The results of these experiments are shown in Table V. The values for all the amino acids except serine and threonine, for each hydrolysis time were averaged and are summarized in the first column. Serine and threonine were calculated by extrapolation to zero time. The values for valine and isoleucine were found to be essentially constant over all times of hydrolysis and were consequently averaged as the other amino acids. The assumed integral values, shown in the second column, are compared to the results of Bocchini (1970). The last two columns report the data calculated on the

[5] R. H. Angeletti and R. A. Bradshaw, manuscript in preparation.

TABLE V: Amino Acid Composition of Nerve Growth Factor.

Amino Acid	Residues[a]/29,000 g of Protein	Assumed Integers	Lit. Values[b]	Residues[a]/14,500 g of Protein	Assumed Integers
Lysine	18.66	19	19	9.33	9
Histidine	7.68	8	8	3.84	4
Arginine	14.74	15	16	7.37	7
Aspartic acid	24.66	25	25	12.33	12
Threonine	28.00[c]	28	30	14.00[c]	14
Serine	22.68[c]	23	23	11.34[c]	11
Glutamic acid	19.36	19	20	9.68	10
Proline	4.74	5	5	2.37	2
Glycine	13.31	13	13	6.65	7
Alanine	18.00	18	19	9.00	9
Valine	27.46	27	29	13.73	14
Half-cystine	11.90[d]	12	10	5.95[d]	
	11.70[e]			5.85[e]	6
Methionine	2.32	2	2	1.16	1
Isoleucine	10.60	11	12	5.30	5
Leucine	8.08	8	8	4.04	4
Tyrosine	4.75	5	4	2.37	2
Phenylalanine	14.40	14	16	7.20	7
Tryptophan	6[f]	6	6	3[f]	3
Total		258	265		127

[a] Values obtained by averaging duplicate analyses for hydrolysates of 24, 48, and 72 hr. [b] Taken from Bocchini (1970). [c] Extrapolated to zero time of hydrolysis. [d] Measured as *S*-carboxymethylcysteine. [e] Measured as cysteic acid (Moore, 1963). [f] Determined spectrophotometrically (Edelhoch, 1967).

basis of a molecular weight of 14,500 and the assumed integral values for the minimal subunit.

Of particular significance is the demonstration by two methods of the presence of 12 residues of half-cystine for the native molecule. *p*-Mercuribenzoate titrations of native NGF in 8 M urea (Boyer, 1954) were carried out to check the oxidation state of these residues. No free sulfhydryl groups could be detected as indicated by the complete absence of a change in absorbance at 255 nm upon the addition of reagent. It may therefore be presumed that the half-cystinyl residues occur as disulfide bridges in the native state.

Discussion

The results described in these experiments suggest that mouse submaxillary NGF is a dimer composed of two identical polypeptide subunits, of molecular weight about 14,500. Sedimentation equilibrium analysis of the native protein in 0.1 N sodium acetate gave a molecular weight of 29,000 ± 1000. However, when the protein was exposed to 6 M Gd-HCl, the molecular weight observed was close to half that observed for the native molecule. Reduced and carboxymethylated protein also gave a molecular weight of about 14,000 in Gd-HCl, indicating that the apparent minimal subunit is not composed of smaller polypeptide chains joined by disulfide cross-links.

Each of the molecular weight values reported was calculated using a partial specific volume of 0.716 ml/g, the value calculated for the native protein from amino acid composition data. If, in fact, the $\bar{v}$ for the NGF samples measured in Gd-HCl is actually lower by 0.02, as has been suggested for this solvent (Hade and Tanford, 1967), values of 12,855 and 14,445 are obtained for the M_w of NGF. Clearly, this degree of change in $\bar{v}$ does not alter the basic observation that native NGF is dissociated into half-molecules in this solvent.

The results of the amino-terminal analysis and the column fingerprint of the tryptic peptides strongly support a dimeric model. The value of 2.3 residues of serine/29,000 molecular weight as amino end group indicates the presence of the two polypeptide chains and lends support to the conclusion that they are of similar or identical structure. The analysis of the column fingerprint, in which 15 of the 35 peptides anticipated on the basis of the lysine and arginine content could be readily identified, further supports the concept of identical subunits. Even more convincing evidence is found in the fact that only six unique half-cystinyl peptides were found, as judged by the distribution of radioactivity, instead of the 12 predicted from the composition of native NGF. In addition, several of these peptides were recovered in micromolar quantities approaching twice the amount of 29,000 molecular weight NGF used in the experiment.

The results obtained in these studies are in excellent agreement with those reported previously. Bocchini and Angeletti (1969) have reported a molecular weight of 30,000 as judged by sedimentation equilibrium, for NGF prepared in the same manner as the material used in these studies, and Zanini *et al.* (1968) found an apparent molecular weight of 28,000 by gel filtration measurements. In addition, these workers also noted that, upon standing in dilute solution, NGF underwent an apparent partial dissociation to produce two species of mol wt 28,000 and 14,000, which were fully separated by gel filtration. Significantly, these two species of NGF were undistinguishable by biological assay and comple-

ment fixation. These results of other workers clearly support the conclusion reached from the data presented above that NGF is composed of identical subunits of about 14,000 and further indicate that the subunit, prepared from dilute solutions is a physiologically active entity.

The most compelling evidence arguing against the existence of identical subunits is the half-cystine content reported by Bocchini (1970). His report that NGF contained 10 residues of half-cystine, all in the disulfide form, is inconsistent with a dimeric model unless the subunits are joined by a disulfide bridge in which each half of the linkage is donated by a half-cystinyl residue in each subunit. The data obtained in the experiments reported here confirm Bocchini's observation that NGF is devoid of free sulfhydryl groups but also eliminate the possibility of an intermolecular bridge. This apparent discrepancy, however, has been eliminated by a reexamination of the amino acid composition. The data presented clearly indicate that NGF contains 12 residues of half-cystine instead of 10, which allow for a structure containing three disulfide bonds per subunit. It should be noted that the remainder of the composition is in good agreement with that reported by Bocchini.

The observation that NGF, with an initial molecular weight of 30,000 is composed of identical subunits of about 14,000 which can apparently exist as stable monomers (Zanini *et al.*, 1968), indicates a marked tendency of these units to associate. In fact, Cohen (1960), who first purified mouse NGF, reported a molecular weight of 44,000 which is in excellent agreement with the molecular weight expected for a trimer and suggests that the molecular weight of NGF may be strongly dependent on the method of preparation. In fact, examination of the whole cell averages for the number-, weight-, and z-average molecular weights for native NGF measured in these studies gave values of 28,000, 29,000, and 32,000, respectively, indicating the presence of small amounts of heavier molecular weight material, possibly corresponding to trace amounts of a trimeric or heavier species.

The determination of the amino end group and isolation of the soluble tryptic peptides represent the first experiments reported on the determination of the chemical structure of mouse NGF. As a means for further clarifying the molecular basis for the mechanism of action of NGF, the determination of the complete primary structure has now been initiated.

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